# Technical Report on the PCR-DGGE Analysis of Bacterial and Fungal Soil Communities

Ver. 3.3

Revised on June 10, 2010

National Institute for Agro-Environmental Sciences

# Table of Contents

For inquiries regarding this manual, please contact:
Seiya Tsushima, Yuko Matsushita
Natural Resources Inventory Center
National Institute for Agro-Environmental Sciences
E-mail: seya@affrc.go.jp, yuhko@affrc.go.jp
Tel.: +81-29-838-8351

# 1. Preparation of soil samples

(1) Pass the collected soil sample through a sieve with a 2-mm mesh size to remove as much of the plant residue as possible. Thoroughly mix the sieved soil in a large plastic bag to avoid variability among the results of replicate soil samples.

(2) Put 0.4 g of the obtained soil in a tube for bead mill homogenization. If DNA is not extracted immediately, store the tubes at -20°C or lower. The sample number from each field should be about 3–5, depending on the characteristics of the target field (i.e., its scale or diversity).

# 2. DNA extraction

DNA is extracted from the soil samples by using the commercially available kit described below. The method includes the process of prevention of DNA adsorption to soil particles by adding skim milk[1)]. The tubes used for bead mill homogenization and test reagents (excluding skim milk) are included in the kit.

【Materials required】

- FastDNA SPIN Kit for Soil (Q-BIOGene)
- Skim milk
- Bead mill homogenizer (FP series from Q-BIOGene, Inc., Bead Smash from Wakenyaku, Co., Ltd., etc.)
- High-speed microcentrifuge

【Extraction procedure】

(1) Add 978 µl of sodium phosphate buffer*[1] and 122 µl of MT buffer to the 0.4 g sample in the tube (Lysing Matrix E) used for bead mill homogenization and suspend the mixture.

*[1] For soil types such as andosol, which strongly adsorb DNA, add 80–160 µl of 20% (w/v) skim milk solution when adding the sodium phosphate buffer. In this case, a

volume equivalent to the skim milk solution must be reduced from the sodium phosphate buffer. Since the optimum amount of skim milk to be added differs from soil to soil, it is necessary to check the optimum amount in advance. To prevent DNA contamination from skim milk, the skim milk solution should be heat-treated in advance at 115°C for 5 min. It is recommended that the skim milk solution be put into tubes (capacity, approximately 2 ml) with screw caps and stored at -20°C.

(2) Set the tube in the bead mill homogenizer. At this time, ensure that the tube cap is tightly closed, since failing to do so may cause the extract to leak. The standard speeds and times for the FastPrep FP series and BeadSmash series are 5.5 m/s for 30 s and 5,000 rpm for 30 s, respectively.

(3) Centrifuge at 14,000 ×*g* for 1 min, and then transfer the supernatant to a new 1.5-ml tube.

(4) Add 250 μl of PPS to the supernatant and mix well by flipping the tube upside down, and then centrifuge at 14,000 ×*g* for 5 min.

(5) Transfer the supernatant to a new 15-ml tube, and add 1 ml of binding matrix suspension to it. Since the binding matrix suspension precipitates easily, mix well to prevent unevenness while dispensing.

(6) Mix gently using a rotator or by hand for 2 min, and then make the tube stand still in a rack until the binding matrix settles.

(7) Remove 500 μl of the supernatant, avoiding removal of the binding matrix.

(8) Suspend the binding matrix again, and transfer 600 μl to a spin filter; then centrifuge at 14,000 ×*g* for 1 min.

(9) Decant the flow-through and pass the remaining suspension through the spin filter using the same procedure, and then collect the binding matrix on the spin filter.

(10) Add 500 μl of salt ethanol wash solution (SEWS-M) to the collected binding matrix, and

then centrifuge at 14,000 ×*g* for 1 min.

(11) Decant the flow-through and centrifuge at 14,000 ×*g* for 2 min to completely remove the SEWS-M, and then transfer the spin filter to a catch tube.

(12) Air-dry the matrix for 5 min, and then add 80 µl*[2] of DES and gently suspend it. At this time, ensure that no damage is caused to the filter by the head of the tip.

*[2] Although the kit manual specifies the use of 50 µl of DES, we recommend 80 µl be used since it is difficult to thoroughly suspend the matrix using 50 µl of DES.

(13) Centrifuge at 14,000 ×*g* for 1 min, and collect the eluate in a catch tube. Confirm DNA extraction by performing agarose gel electrophoresis by using approximately 5–10 µl of the eluted DNA solution*[3]. If the DNA solution is not used immediately, freeze-store it at a temperature of -20°C or lower.

*[3] When humic soil is used, the solution appears to be slightly colored. In case PCR inhibition occurs, additional purification must be performed using a commercially available DNA purification column etc.

# 3. PCR

## 3-1. Bacteria

Use the following primer set[2)], which targets the V6-8 variable region of eubacterial *16S rRNA* genes.

Forward primer: F984GC

5′-cgc ccg ggg cgc gcc ccg ggc ggg gcg ggg gca cgg ggg gaa cgc gaa gaa cct tac-3′

The underlined segment indicates a GC clamp.

Reverse primer: R1378

5′-cgg tgt gta caa ggc ccg gga acg-3′

【PCR conditions】

Use TOYOBO “KOD-Plus-” under the following conditions:

- The composition of the reaction mixture (Total volume, 50 μl)

| | Amount | Final concentration |
|---|---|---|
| Sterile distilled water | 33 μl | |
| 10× buffer | 5 μl | 1× |
| dNTPs (2 mM) | 5 μl | 0.2 mM each |
| $MgSO_4$ (25 mM) | 2 μl | 1 mM |
| F984GC (10 μM) | 1 μl | 0.2 μM |
| R1378 (10 μM) | 1 μl | 0.2 μM |
| BSA (20 mg/ml)*[4] | 1 μl | 0.4 μg/μl |
| KOD-Plus- | 1 μl | |
| Template DNA*[5] | 1 μl | |

*[4] Bovine serum albumin solution (BSA; TaKaRa #2320). The inhibition of PCR, which may be caused by humic substances, can be prevented by the addition of BSA.

*[5] Add 1 μl of the DNA solution used in the extraction method described above. For the negative control experiment, the mixture without the template DNA must be prepared.

- Reaction cycle

94°C (2 min) → [94°C (15 s), 55°C (30 s), 68°C (30 s)] × 34

【Detection and purification of PCR product】

To confirm that PCR product of the expected size has been obtained (approximately 470–480 bp), perform agarose gel electrophoresis using approximately 2–3 μl of the PCR product. Purify the remaining product using a commercially available PCR product purification kit (QIAGEN QIAquick PCR Purification Kit etc.), and then measure the DNA concentration. If denaturing gradient gel electrophoresis (DGGE) is not performed immediately, store the purified product at -20°C or lower.

## 3-2. Fungi

Use the following primer set[3], which targets fungal *18S rRNA* genes.

Forward primer: NS1

5′-gta gtc ata tgc ttg tct c-3′

Reverse primer: GCFung

5′-cgc ccg ccg cgc ccc gcg ccc ggc ccg ccg ccc ccg ccc cat tcc ccg tta ccc gtt g-3′

The underlined segment indicates a GC clamp.

【PCR conditions】

Use TOYOBO “KOD-Plus-” under the following conditions:

- The composition of the reaction mixture (Total volume, 50 μl)

| | Amount | Final concentration |
|---|---|---|
| Sterile distilled water | 32 μl | |
| 10× buffer | 5 μl | 1× |
| dNTPs (2 mM) | 5 μl | 0.2 mM each |
| $MgSO_4$ (25 mM) | 2 μl | 1 mM |
| NS1 (10 μM) | 1.5 μl | 0.3 μM |
| GCFung (10 μM) | 1.5 μl | 0.3 μM |
| BSA (20 mg/ml)*[6] | 1 μl | 0.4 μg/μl |
| KOD-Plus- | 1 μl | |
| Template DNA*[7] | 1 μl | |

*[6] Bovine serum albumin solution (BSA; TaKaRa). The inhibition of PCR, which may be caused by humic substances, can be prevented by the addition of BSA.

*[7] Add 1 μl of the DNA solution used in the extraction method described above. For the negative control experiment, the mixture without the template DNA must be prepared.

- Reaction cycle

94°C (2 min) → [94°C (15 s), 50°C (30 s), 68°C (30 s)] × 30

【Detection and purification of PCR product】

Perform agarose gel electrophoresis using an approximately 2- to 3-µl aliquot of the PCR product to confirm that a product of the expected size (approximately 390 bp) has been obtained. Purify the remaining product using the same method used for common bacterial analysis, and then measure the DNA concentration. If DGGE is not performed immediately, store the purified product at -20°C or lower.

## 4. DGGE

Bio-Rad DCode system is used for DGGE. The procedure is the same as that for bacteria and fungi except for the electrophoresis conditions shown in Tables 1 and 2.

Table 1 Electrophoresis conditions

| Condition | For bacteria | For fungi |
|---|---|---|
| Acrylamide/bis concentration | 6% | 7% |
| Denaturing concentration gradient | 50% → 70% | 20% → 45% |
| Temperature | 58°C | 60°C |
| Voltage | 50 V | 50 V |
| Time | 18 h | 20 h |

Table 2 Composition of the gel stock solution for DGGE*[8]

| Composition | For bacteria | | For fungi | |
|---|---|---|---|---|
| | 50% denaturing solution | 70% denaturing solution | 20% denaturing solution | 45% denaturing solution |
| 40% acrylamide/bis 37.5:1 | 15 ml | 15 ml | 17.5 ml | 17.5 ml |
| 50× TAE | 2 ml | 2 ml | 2 ml | 2 ml |
| Formamide (deionized) | 20 ml | 28 ml | 8 ml | 18 ml |
| Urea | 21 g | 29.4 g | 8.4 g | 18.9 g |
| Ultrapure water | up to 100 ml | up to 100 ml | up to 100 ml | up to 100 ml |

*[8] 100% denaturing solution is equivalent to a combination of 7 M urea and 40% formamide.

【Materials required】

- DCode system (Bio-Rad)

(Main unit, casting stand, sandwich clamp, glass plate, spacer, syringes, tubes, needles, Y-fitting, comb, and gradient former)

- Power supply
- Suction filter (IWAKI membrane filter system #8110-250 etc.)
- Vessel used for gel staining (polypropylene)
- Gel imaging equipment
- 40% acrylamide/bis 37.5:1 solution
- 50× TAE
- Formamide (deionized)
- Urea (molecular biology grade)
- Ammonium persulfate
- TEMED
- Dye solution (see manual attached to DCode)
- 2× loading dye (see manual attached to DCode)
- SYBR Green I (TaKaRa #F0513 etc.)

【Preparation of the stock solution for DGGE】

(1) On the basis of the composition given in Table 2, prepare 2 kinds of gel stock solutions with different denaturation concentrations. It is possible to cast the DGGE gel for 6 sheets using 100 ml of each stock solution. Unpolymerized acrylamide is toxic. Wear rubber gloves when handling it.

(2) Filter the prepared solution using the suction filter to remove impurities.

(3) Store the solution in a light-resistant bottle at 4°C. Use within approximately 1 month*[9].

*[9] Since formamide is gradually degraded by moisture, the ability of DNA denaturation will be reduced in the deteriorated gel stock, with the band declining toward the downstream side. If this is observed, replace the stock solution regardless of the

preservation period.

【Casting Gels】

(1) Assemble the plate following the instructions in the DCode manual*[10]. Secure the plate on the casting stand, and tape the tube used for gel injection in the center of the plate.

*[10] Ensure that the spacer and the edge of the glass plate are on a flat plane to prevent gel leakage.

(2) Prepare an adequate amount of 10% ammonium persulfate solution*[11].

*[11] Use approximately 300 µl per gel sheet. Prepare just before use.

(3) Pour 16 ml of each denatured gel stock solution into clean beakers etc.

(4) Add 100 µl of dye solution to the highly denaturing gel stock solution.

(5) Add 144 µl of ammonium persulfate solution prepared in Step (2) to both stock solutions and lightly shake.

(6) Add 14.4 µl of TEMED to both stock solutions, lightly shake, and suction the whole amount of each solution into syringes with an attached tube. Remove air from inside the syringes and tubes, set the syringes on a gradient former*[12], and then connect the edge of each tube to the tube used for gel injection with a Y-fitting.

*[12] Ensure not to confuse the left and right of the gradient former. Firmly secure a highly denaturing gel stock solution syringe on the right-hand side of the wheel and a slightly denaturing gel stock solution syringe on the left-hand side. If they are not tightly secured with screws, the syringe will become misaligned at the time of injection, thereby causing variation in gel quantity. Rotate, in advance, the wheel of the gradient former in the reverse direction of gel injection operation until it cannot be rotated anymore.

(7) Slowly rotate the wheel of the gradient former toward yourself at a steady speed. Inject the solution until it reaches the upper edge of the plate, disconnect the tubes, and then insert the comb taking care to prevent the entry of air bubbles. Return the solution remaining in the

tubes and syringes to the container, and dispose of it after polymerization. Rinse the tubes and syringes with distilled water so that acrylamide will not set in them.

(8) Polymerize for 3 h or longer.

Since polymerization of acrylamide starts as soon as TEMED is added, steps (6)–(8) must be performed quickly for each gel. Because the polymerization of acrylamide progresses rapidly at a higher temperature, chill the gel stock solution well, in advance. If there is a large difference in temperature between the glass plate and the stock solution, polymerization will not progress uniformly, thereby causing distortion of the denaturing gradient. It is recommended that, if possible, gel casting be performed in a low-temperature room in order to keep the glass plate at a low temperature. In this case, polymerization will take longer (Return the product to room temperature after it has been solidified to a certain degree).

【Electrophoresis】

(1) Prepare 7 L of 1× TAE buffer, and pour it into the electrophoresis tank until it reaches between the “Fill” and “Run” positions. Turn on the heater, and set the temperature approximately 5°C higher than the electrophoresing temperature*[13]. Perform this step while gel polymerization is in progress, because it takes a long time for the buffer to be heated to an appropriate temperature.

*[13] This is because the buffer temperature drops when the gel slabs are set later.

(2) Carefully pull the comb out of the sufficiently polymerized gel. Thoroughly remove any extra gel from the plate surface, and cleanse the wells using an injector etc. Further, straighten the border of the wells.

(3) Attach the gel slab to the core of DCode, and set it on the electrophoresis tank. At this time, if the entire core is sunk, the buffer will overflow. Therefore, put aside approximately 300 ml of the buffer from the electrophoresis tank in a separate vessel in advance, and after setting the core, return the buffer in the separate vessel to the upper part of the core. DCode enables electrophoresis with 2 gels simultaneously, but even when electrophoresing just one gel, set the plates on both surfaces. In this case, you can use plates without a gel and spacers.

(4) Start the pump and fill the electrophoresis tank with the buffer to the “Maximum” line. Check to see that the upper buffer retains a fixed water level as the pump is operated. Set the temperature to approximately 2°C higher than the temperature during electrophoresis*[14], and wait until the temperature stabilizes.

*[14] This is because the buffer temperature drops during sample application.

(5) Prepare a sample for electrophoresis by mixing an equivalent of 200 ng (when a 16-well comb is used) of PCR product with the same quantity of 2× loading dye.

(6) Remove the upper unit, and apply the sample to each well. It is better not to use the wells at the ends since they are likely to cause distortion in electrophoresis. Prepare marker lanes*[15] to compare the movement patterns between the different gels.

*[15] Use of DGGE Marker III for bacteria and DGGE Marker IV for fungi is recommended (both manufactured by Nippon Gene Co., Ltd.).

(7) Set the temperature given under the electrophoresis conditions in Table 1, and start the pump. At this time, if the buffer level does not reach the “Maximum” line of the electrophoresis tank, replenish the buffer. After the temperature stabilizes, start electrophoresis in accordance with the electrophoresis conditions shown in Table 1.

【Staining】

Although SYBR Green I used in the following procedure is considered less harmful than ethidium bromide, wear rubber gloves for safety during the staining operation. The gloves also prevent gels from being soiled with sebum etc.

(1) Dissolve the core and remove the sandwich clamp from the glass plate.

(2) Remove the smaller glass plate from the gel by slowly and carefully lifting the spacer along the edge.

(3) Carefully remove the gel from the larger glass plate, and transfer it to a vessel used for staining. At this time, take care not to damage the gel.

(4) Lightly rinse the gel with an appropriate quantity of 1× TAE buffer, and discard the buffer.

(5) Add 100 ml of 1× TAE buffer and 10 µl of SYBR Green I, and gently shake on a turntable etc., for 30 min. Read the manual for SYBR Green I thoroughly before handling it. Since SYBR Green I is adsorbed to glass and polyethylene, use a vessel made of polypropylene for staining.

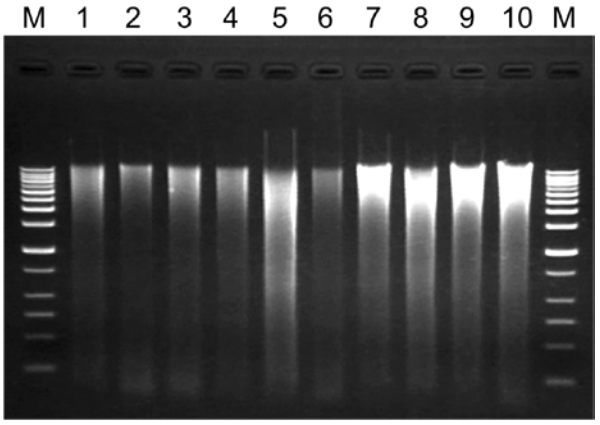
(6) Generate an image of the electrophoretic profile following the instructions in the operation manual for the imaging equipment used. The use of a large-size gel image scanner is ideal, but by using a transilluminator together with a digital camera that has a manual exposure function, it is possible to obtain a good electrophoretic profile if appropriate adjustments are made. Minigel imaging equipment and Polaroid cameras usually lack resolution.

# 5. Examples

## 1. The extraction of DNA from the soil samples

Samples

1 Field soil (andosol)
2 Field soil (yellow soil)
3 Field soil (gray lowland soil)
4 Field soil (low humic andosol)
5 Greenhouse floor soil (low humic andosol)
6 Field soil (low humic andosol)
7 Field soil (brown lowland soil)
8 Field soil (low humic andosol)
9 Paddy field (andosol)
10 Paddy field (gray lowland soil)
M Kb DNA ladder (Stratagene)



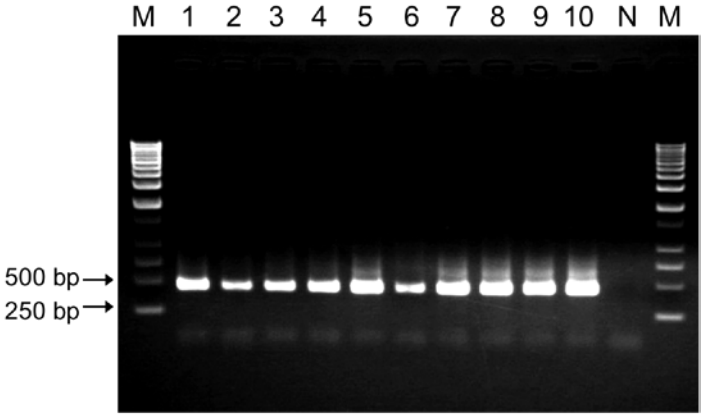
1% agarose gel electrophoresis at 100 V for 30 min

In accordance with this manual, 80 µl of DNA solution was extracted from 0.4 g of each soil sample, 6 µl of which was used for agarose gel electrophoresis. On extraction, 80 µl of 20% skim milk solution was used for samples 2 and 3, 120 µl for sample 1, and 160 µl for samples 4, 5, 6, and 8.

## 2. PCR

【PCR of bacterial 16S rDNA】

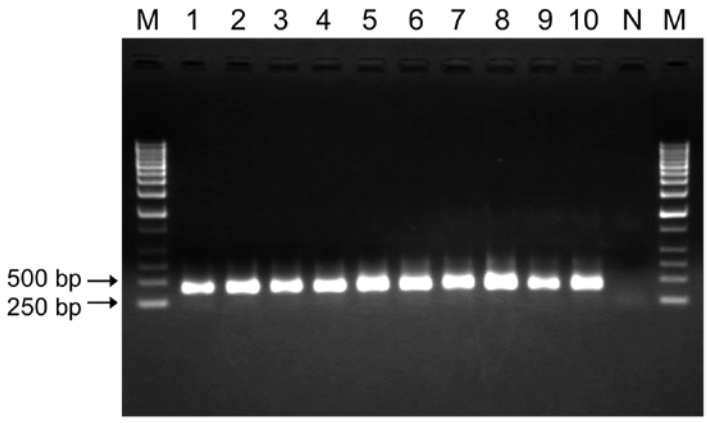
Using the DNA extracted from the abovementioned soil samples as a template, PCR amplification of bacterial 16S rDNA was performed. Two microliters of each product was used for agarose gel electrophoresis. N is the negative control.



1% agarose gel electrophoresis at 100 V for 30 min

【PCR of fungal 18S rDNA】

Using the DNA extracted from the abovementioned soil samples as a template, PCR amplification of fungal 18S rDNA was performed. Two microliters of each product was used for agarose gel electrophoresis. N is the negative control.




1% agarose gel electrophoresis at 100 V for 30 min

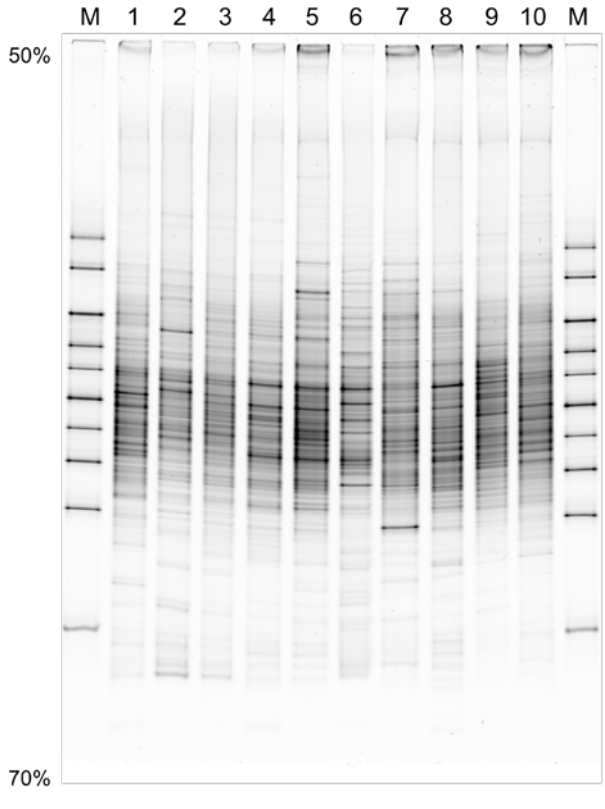
## 3. DGGE

【Bacteria】

After purifying the bacterial 16S rDNA PCR products, an equivalent of 200 ng of each product was used for electrophoresis under the conditions specified in this manual.

After staining with SYBR Green I, imaging was performed using PharosFX (Bio-Rad).

M is a marker optimized for the analysis conditions (DGGE Marker III; Nippon Gene Co., Ltd.).



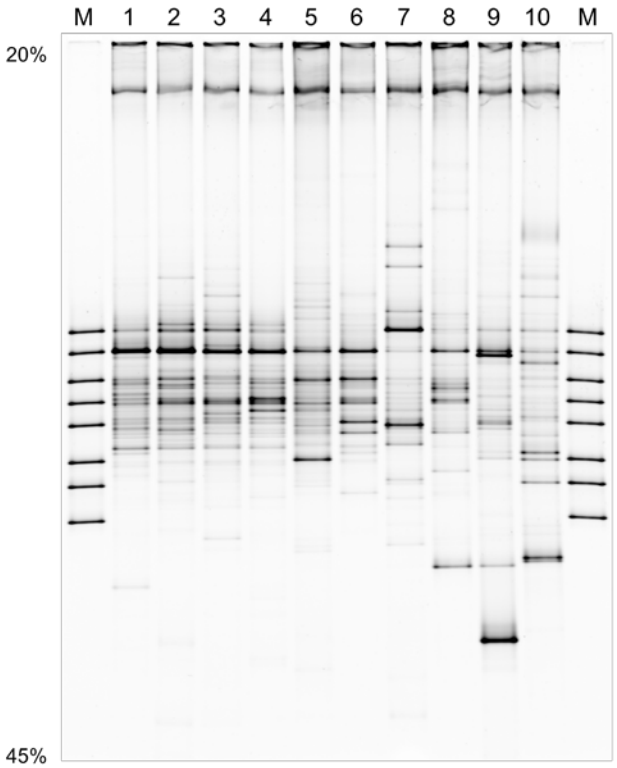

6% polyacrylamide gel electrophoresis at 58ºC at 50 V for 18 h

【Fungi】

After purifying the fungal 18S rDNA PCR products, an equivalent of 200 ng of each product was used for electrophoresis under the conditions specified in this manual.

After staining with SYBR Green I, imaging was performed using PharosFX (Bio-Rad).

M is a marker optimized for the analysis conditions (DGGE Marker IV; Nippon Gene Co., Ltd.).




7% polyacrylamide gel electrophoresis at 60ºC at 50 V for 20 h

# 6. Troubleshooting

## 【Problems related to DNA extraction】

-DNA cannot be extracted, or the extracted amounts are extremely small.

→ Change the amount of skim milk added. Depending on the soil, the yield of DNA may be low because the original biomass is also low.

-Spin filter is broken and binding matrix falls off.

→ Depending on the soil, this trouble may happen frequently. If this happens, collect the matrix that has fallen off, suspend it in SEWS-M, and transfer it to a new filter.

-The extracted DNA solution is colored.

→ When highly humic soil is used, the extracted DNA solution may have a light brown color. If PCR is inhibited, re-purify the DNA solution using a commercially available DNA purification kit etc.

-The sample diffuses during the electrophoresis using the agarose gel.

→ This may be because there is ethanol remaining in the sample. The binding matrix is not sufficiently dried after it is cleaned with SEWS-M.

## 【Problems related to PCR】

-No amplification is obtained.

→ When only a particular sample is not amplified, this problem is probably caused by insufficient amount of template DNA or the presence of PCR-inhibiting contaminants (humic substances) in the DNA solution. If the DNA concentration is clearly low, change the amount of skim milk that may improve the extraction efficiency. If a sufficient amount of DNA is not obtained even after implementing this measure, increase the amount of template DNA or the cycle number. If the DNA solution is clearly colored, purify it using a commercial DNA purification kit etc.

→ Reagents used for PCR should be completely dissolved and sufficiently mixed before use.

→ Confirm the composition of the reaction mixture and the primer concentration.

-There are many nonspecific products.

→ Ensure that excessive amounts of polymerase and $Mg^{2+}$ are not present. Take maximum care to prevent the polymerase, which is a highly viscous solution, from adhering to the pipette tips.

→ If the template DNA concentration is extremely high, dilute it before use.

-Amplification is observed in the negative control.

→ If the cause cannot be clearly identified, it is better to use new reagents. Since a large quantity of PCR product is treated in the series of PCR-DGGE work, maximum care should be taken to prevent contamination of the PCR product. The use of tips with filters is recommended for the pipetting steps in PCR.

## 【Problems related to DGGE】

-Acrylamide clots during gel casting.

→ Polymerization progresses rapidly if the temperature of the gel stock solution is high. Chill the stock solution well before use.

-Gels do not reach the upper edge of the plate even if the gradient former is rotated all the way.

→ When assembling the glass plate, if the bottom surface is not flat, gels may leak during injection.

→ If the screws used to fix the syringe to the gradient former are not fully tightened, the syringe itself is moved aside when the wheel is rotated and the piston is not fully pushed in. In particular, this trouble is likely to happen if the syringe rubber plunger generates large friction due to deterioration. In this case, replace the syringe with a new one.

-The electrophoretic profile is mottled with dirt.

→ It is important to keep the instruments, particularly the glass plates, clean. Ensure that dust does not adhere to the glass plates when assembling. Avoid wiping the glass plate with paper that is likely to leave fibers.

→ If the gel stock itself contains impurities, refilter the stock.

→ Ensure that the buffer and equipment used for staining and imaging capture are not dirty.

-Bands appear as a smear.

- → Because of the structural characteristics of DCode, the bands on the back side gel tend to be blurry. This problem can be improved by adding a magnetic stirrer to the electrophoresis tank to stir the buffer during electrophoresis.
- → If a sample that has been stored under poor conditions is electrophoresed, the band may smear.
- → If the timing of comb insertion is delayed, or air bubbles adhere to the comb, the wells may become irregular, thereby affecting the electrophoresis profile.
- → When injecting the sample to the well, take maximum care not to let the sample diffuse.

-The pattern is distorted.

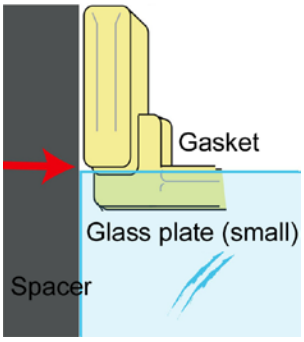
- → If the polymerization of acrylamide progresses rapidly, the denaturing gradient tends to be distorted. To prevent the pattern from being distorted, chill the glass plate and the gel solution in advance so that polymerization progresses slowly.

-The pattern reproducibility is poor.

- → Since the deterioration of the syringe rubber plunger greatly influences pattern reproducibility, the damaged syringes must be replaced immediately.
- → Use of old gel stock or reuse of TAE buffer is not recommended for good reproducibility.
- → Use deionized formamide. If formamide appears to be colored, it needs to be deionized again. To deionize formamide, add 10 g of AG501 X-8 resin (Bio-Rad) to 100 ml of formamide, stir for 1 h, and filter.
- → If friction is experienced on the wheel of the gradient former, adjustment is necessary. It may be improved by applying silicon spray to the part where friction is generated, but if disassembly is necessary for adjustment, it is better to request the manufacturer.

-Upper buffer falls off.

- → Ensure that the gasket is sandwiched between the core and the plate with no space in between. There tends to be a gap at the position marked by the red arrow in the diagram.



-The stirring shaft touches the plate, thereby making a noise.

→ This is because the assembly of the core and the plate is incomplete, or the shaft is moved aside. In the latter case, if the problem is not solved by adjusting the position, repair is necessary. If electrophoresis is performed with the shaft touching the plate, the plate will vibrate, thereby disturbing the electrophoresis profile.

# 7. References


1) Hoshino TY and Matsumoto N. 2004. An improved DNA extraction method using skim milk from soils that strongly adsorb DNA. Microbes and Environments, 19(1), 13-19.

2) Heuer H, Kresk M, Baker P, Smalla K, and Wellington EM. 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Applied and Environmental Microbiology, 63(8), 3233-3241.

3) May LA, Smiley B, and Schmidt MG. 2001. Comparative denaturing gradient gel electrophoresis analysis of fungal communities associated with whole plant corn silage. Canadian Journal of Microbiology, 47, 829-841.